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Purification and Partial Characterization of an Autotomy-Promoting Factor from the Sea Star *Pycnopodia helianthoides*

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Abstract. Echinoderms possess collagenous connective tissues that are capable of rapid, nervously mediated changes in their tensile strength. Arm autotomy in sea stars is facilitated by a rapid decrease in the tensile strength of connective tissues in the arm base. In this study, an autotomy-promoting factor (APF) has been isolated from the fluids released by scalded or autotomizing sea stars (*Pycnopodia helianthoides*). When injected into the coleom, APF elicits a complex behavioral response that culminates within minutes in multiple arm autotomy and a generalized softening of the body wall. Injection of fluid from intact, untreated sea stars does not promote the autotomy response. APF is a water soluble, heat-labile substance derived from the body wall. It is ammonium sulphate precipitable and its activity is reduced or destroyed by several proteolytic enzymes. On the basis of its gel permeation elution pattern, APF has M_r of about 1200 Daltons. APF can be purified to a single peak of activity by reversed-phase HPLC. We conclude the substance is a peptide or has a peptide component.

Introduction

Echinoderms are unique in their possession of collagenous connective tissues that are capable of rapid, nervously mediated changes in their tensile strength (Motokawa, 1984; Wilkie, 1984). Although such mutable collagenous tissues are involved in many aspects of echinoderm biology (Emson, 1985; Wilkie and Emson, 1987; Motokawa, 1988; Wilkie, 1988), the most spectacular example of their role is in the phenomenon of autotomy, or self-mutilation. When the tensile strength of key collagenous tissues decreases rapidly and irreversibly, the result can be arm autotomy, evisceration (a complex form of autotomy in some sea cucumbers resulting in the expulsion of the viscera), or fission (a form of asexual proliferation in which the body splits into two segments).

Many sea stars will rapidly autotomize arms in response to disturbance or damage (Emson and Wilkie, 1980). Arm autotomy can be induced in the laboratory by handling, exposure to air, wounding, ligation, amputation of the tube feet, electrical shock, and chemical stimuli. Breakage usually occurs at the base of the arm, although in a few species it can occur at any level. In nature, arm autotomy probably has a defensive function by effecting release from predators such as crabs and other sea stars (Hancock, 1955, 1974). Sea stars also autotomize arms that are heavily parasitized and thus, perhaps, use autotomy as a mechanism to reduce parasite load (Riggenbach, 1903).

Chaet (1962), in the course of research on invertebrate thermal toxins, provided the first evidence that endogenous chemical factors promote autotomy in sea stars. He discovered that *Asterias forbesi* would autotomize its arms when injected with the fluid exuded by scalded conspecifics, but not when injected with fluid from non-scalded donors. He found the factor responsible for autotomy was a heat-stable, dialyzable molecule derived, apparently, from the lining of the coelomic cavity. Ob-

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servations by Davis (1967) that the parasitic gastropod, *Stylifer linckiae*, suppressed arm autotomy in the sea star, *Linckia multifora*, provide alternative, indirect evidence that arm autotomy in sea stars may be under some form of chemical control.

Chemical factors have also been implicated in the control of evisceration in sea cucumbers, a complex form of autotomy in which viscera are expelled through a rupture in the body wall. The coelomic fluid expelled by eviscerating *Sclerodactyla (Thyone) briareus* (Smith and Greenberg, 1973) and *Eupentacta quinquesemita* (Byrne, 1986) contains a factor that promotes evisceration when injected into conspecifics. Potential sources of this factor are the haemal and peritoneal systems. Smith and Greenberg (1973) suggested that the *S. briareus* evisceration factor is a small molecule on the basis of preliminary gel permeation chromatography experiments.

As Wilkie (1984) has emphasized, it is difficult to assess the physiological significance of autotomy-inducing factors in echinoderms until more is known about their chemical nature, source, site of activity, and mode of action. To begin to address some of these problems, we have undertaken a study of the chemical control of arm autotomy in the sea star, *Pycnopodia helianthoides* (Brandt 1835), a large, multi-armed species from the west coast of North America that readily autotomizes arms (Lambert, 1981).

Materials and Methods

Materials

Sea stars (*Pycnopodia helianthoides*) that ranged in size from 5 to 35 cm in overall diameter were collected by diving in Saanich Inlet near Victoria, British Columbia, Canada, and maintained at 11°C in a closed seawater system at the University of Victoria. Both male and female sea stars were used, always within a week of collection.

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri). Sephadex gels (G-25) were purchased from Pharmacia (Canada) Inc. (Baie D'Urfé, Québec). Supelcosil C_{18} columns were purchased from Supelco Canada Ltd. (Oakville, Ontario). HPLC grade trifluoracetic acid and acetonitrile were purchased from Pierce Chemical Co. (Rockford, Illinois). Artificial seawater (ASW) consisted of Jamarin U (Jamarin Laboratory, Osaka, Japan).

Preparation of crude extracts

Crude extracts of sea star autotomy-promoting factor (APF) were obtained with slight modification of Chaet's (1962) procedure. Large (25–35 cm overall diameter) sea stars were placed individually in dry 12 × 24 inch plastic autoclave bags (VWR Scientific, San Francisco, California) and immersed for 1–1.5 min in water (76°C). During immersion, the body wall of the sea star became very soft and some of the arms were autotomized. Fluid of mainly coelomic origin was released into the bag through tears that developed in the softened body wall and from wounds resulting from autotomy. This fluid was collected in chilled beakers and centrifuged at 2000 × *g* for 10 min. The pellet was discarded and the supernatant decanted and either bioassayed immediately for APF activity or stored (−20°C or freeze-dried). Control fluid consisted of coelomic fluid withdrawn by syringe from untreated, intact animals.

Fluid from autotomizing sea stars was also collected and tested for APF activity. Large sea stars were placed individually in autoclave bags and dropped to the laboratory floor from a height of 2 m to induce them to autotomize many of their arms. The fluid released into the bag during autotomy was collected and centrifuged and the supernatant retained for bioassay or storage.

To determine the general source of APF activity in *P. helianthoides*, the coelomic fluid was first drained (by cutting off the tips of 2–3 arms) from five sea stars (20 cm in overall diameter), and the animals quickly dissected into body wall, pyloric caeca, and stomach components (gonads were not present in these sea stars). The coelomic fluid (pooled volume approximately 500 ml) was poured into an autoclave bag, immersed in water (76°C) for 1.5 min, cooled, and bioassayed. The tissues were placed separately in autoclave bags, with a small amount of ASW, immersed in water (76°C) for 1.5 min, and the resulting extracts collected, cooled, and bioassayed.

Bioassay

Test samples and control fluids were bioassayed by intracoelomic injection into 3–6 smaller (5–10 g wet weight), intact recipient sea stars held in dishes of seawater. The amount injected was 150 μl/g wet weight of sea star (0.75 to 1.5 ml per individual). Injections were made into 3–4 different arms of the sea star. The criterion for a positive response was loss of one or more arms within 10 min of injection.

Treatment with proteolytic enzymes

Samples of crude APF were incubated for 1h at 37°C with either trypsin (100 μg/ml, pH 8.0), protease K (100 μg/ml, pH 8.0) or pronase (10 mg/ml, pH 7.2) and then bioassayed for APF activity. Controls were constructed by: (1) substituting artificial seawater for the test sample, or (2) using samples of crude APF incubated without enzyme.

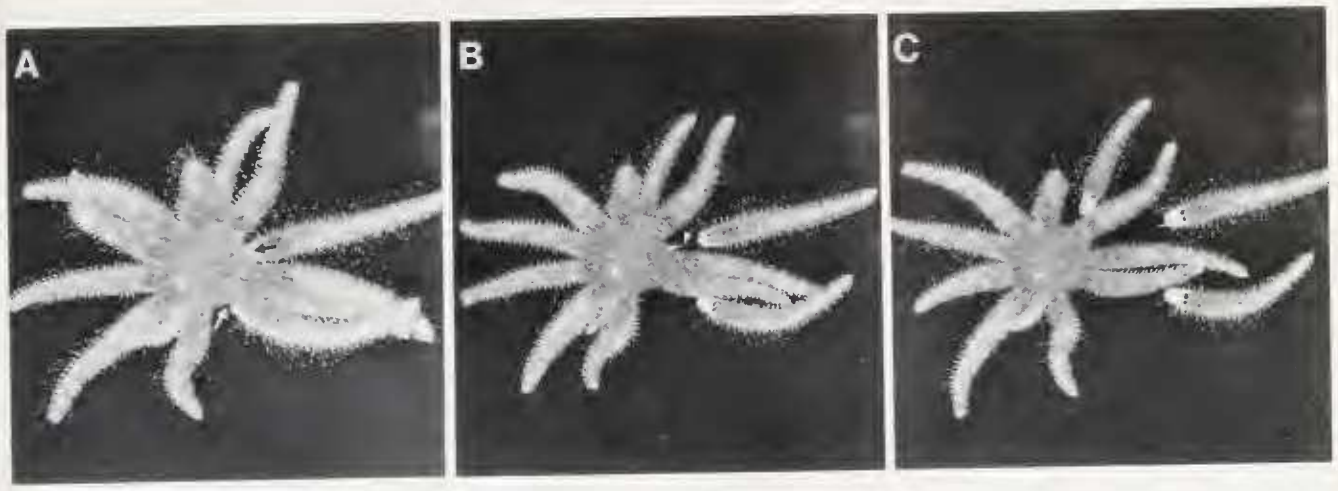

Figure 1. Response to intracoelomic injection of crude autotomy-promoting factor (APF). Onset of autotomy is apparent at the base of 2 arms (arrows) after 25 s (A); after 45 s 1 arm has autotomized completely, while the second arm remains attached to the disc by the pyloric duct only (arrowhead) (B); 3 arms have autotomized completely after 60 s and begun to crawl away from the rest of the sea star (C). ×0.5.

Purification

Gel permeation chromatography. Samples of crude APF were concentrated by freeze-drying and chromatographed on a column of Sephadex G-25 with 0.05 *M* NaCl as the mobile phase. Fractions (5 ml) were collected, pooled to 15 ml, freeze-dried, redissolved in ASW, and bioassayed. Protein concentration in the pooled fractions was estimated by the method of Lowry *et al.* (1951).

High performance liquid chromatography (HPLC). Fractions from the Sephadex G-25 column with APF activity were further analyzed by reversed-phase HPLC. This was performed on a Varian 5000 liquid chromatograph with a 1-ml sample loop, 254-nm UV absorption detector, and Supelcosil LC-18-DB column (250 mm × 4.6 mm, C_{18}, 5 μm particles). The solvent system consisted of a gradient of 0.1% trifluoracetic acid (aqueous phase) and 0.1% trifluoracetic acid in 70% acetonitrile (organic phase). Fractions (4 ml) were collected, freeze-dried, redissolved in ASW, and bioassayed.

Results

Response to crude APF

Injection of a sample of crude APF into an intact sea star (*P. helianthoides*) elicits a complex behavioral response (Fig. 1). Initially, waves of swelling travel through the body of the sea star causing transient and often localized distension of the body wall, particularly at the arm tips. A brief period of immobility often follows, during which the sea star attaches itself securely to the substratum with the tube feet. One or more arms then start to pull away from the disc as a result of centripetal movements of the tube feet. Simultaneously, the body wall tissues at the base of these arms become soft and transparent and begin to stretch apart as the arms pull away from the disc. The separation of the arm and its contents from the disc occurs in a plane passing anywhere between about the 2nd and 18th pairs of ambulacral ossicles, with larger animals autotomizing arms more distally. The tissues of the aboral body wall rupture first, then those binding the ambulacral ossicles; the pyloric duct ruptures last. By the completion of autotomy, the entire body wall of the sea star is irreversibly softened, and morbidity ensues.

Crude APF derived from heat-treated or autotomizing sea stars was equally effective in promoting arm autotomy, eliciting a full response in about 70% of the sea stars bioassayed (Fig. 2). Those sea stars that did not actually autotomize an arm invariably exhibited a strong response to injection of the crude APF that included swelling, pulling movements of the arms, and constriction of the arm bases. Mean time to initial arm autotomy in individuals injected with crude APF from heat-treated and autotomizing sea stars was 5.1 min (S.E.M. = 0.5 min, n = 10) and 19.4 min (S.E.M. = 9.4 min, n = 5), respectively. Injections of coelomic fluid withdrawn from normal, untreated sea stars never promoted autotomy (Fig. 2).

Source

APF activity was detected in body wall extracts, with 4 of the 5 sea stars bioassayed autotomizing arms within 8 min. APF activity was not present in extracts of pyloric

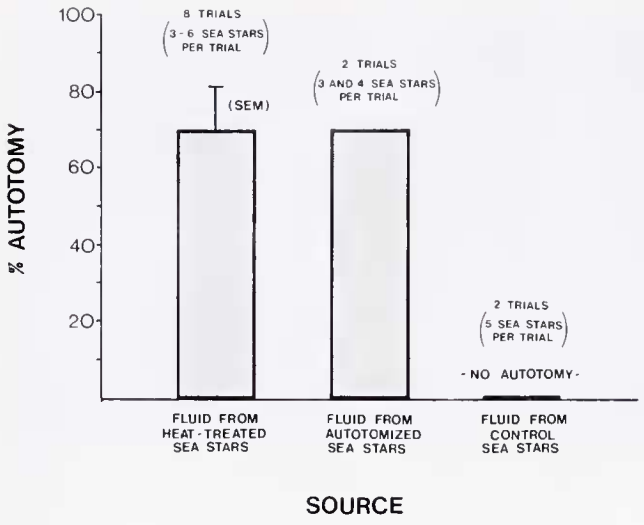

Figure 2. Results of bioassay by intracoelomic injection of fluids from heat-treated, autotomizing, and control sea stars.

caecum and stomach, and not in heated coelomic fluid (5 sea stars bioassayed for each treatment). This indicates that APF is derived from one or more of the tissues comprising the body wall.

Chemical nature of APF

pH. Crude APF from scalded sea stars was pH 6.7 (n = 4). Fluid obtained from autotomizing sea stars was pH 7.2 (n = 3). Coelomic fluid withdrawn from untreated, intact sea stars was pH 7.4 (n = 3).

Solubility. Partitioning of crude APF between aqueous and organic solvents (1 part sample in distilled water: 7 parts methanol:13 parts chloroform) resulted in APF activity in the aqueous phase but not in the organic phase or the interphase. This suggests that APF is a polar molecule and that it is not a lipid or a lipoprotein.

Stability. No activity was observed in a sample of crude APF boiled for 10 min, cooled to 4°C, and bioassayed. An aqueous sample of crude APF lost activity overnight at 4°C. Samples of freeze-dried crude APF lost activity after a few days at room temperature, but retained activity for several weeks at −20°C.

Ammonium sulphate precipitation. Precipitates of crude APF in 50% ammonium sulphate were redissolved in ASW and showed APF activity when bioassayed. Mean time to initial arm autotomy was 2.5 min (S.E.M. = 1.8 min, n = 4).

Treatment with proteolytic enzymes. Incubation of crude APF with trypsin at 100 μg/ml resulted in a smaller proportion of positive bioassays relative to the control (Table I). Incubation with protease K at 100 μg/ml also resulted in a reduced number of positive bioassays as well as a significantly longer time to initial arm autotomy (Mann-Whitney test, $P < 0.001$) in the sea stars that did autotomize arms (Table I). Incubation with pronase at 10 mg/ml completely abolished APF activity. Enzymes in ASW did not promote autotomy, while samples of crude APF incubated without enzyme retained APF activity (Table I). Thus, proteolytic enzymes reduced or abolished APF activity.

Gel permeation chromatography

Fractions with APF activity eluted just after vitamin B_{12} (MW = 1355) and before angiotensin II (MW = 1046) in a column of Sephadex G-25 (Fig. 3). The mean time to initial arm autotomy following injection with these partially purified fractions was 0.39 min (S.E.M. = 0.06 min, n = 4), considerably shorter than the response time following injection with crude APF (see above). Spectrophotometric absorbance maxima in fractions with APF activity were at 220 and 254 nm.

HPLC

Further fractionation of active fractions from the Sephadex G-25 column by reversed phase HPLC yielded a series of peaks. Only one of these peaks, eluting consistently at 45 to 48 min, had APF activity (Fig. 4).

Discussion

We have shown that an autotomy-promoting factor (APF) is present in the body fluids of both scalded and autotomizing *Pycnopodia helianthoides.* APF injected into the coelom of intact *P. helianthoides* elicits a com-

Table I

Bioassay results of treatment of crude APF with proteolytic enzymes

Treatments	% Autotomy (n = 5)	Time (min) (mean ± S.E.M.)
Crude APF (pH 7.0)[1]	100	4.8 ± 0.8
Crude APF (pH 8.0)[1]	100	7.1 ± 1.0
Crude APF + trypsin (100 μg/ml, pH 8.0)	60	8.6 ± 1.4
Crude APF + protease K (100 μg/ml, pH 8.0)	40	14.7 ± 2.8
Crude APF + pronase (10 mg/ml, pH 7.2)	0	no autotomy
ASW + trypsin (100 μg/ml, pH 8.0)[2]	0	no autotomy
ASW + protease K (100 μg/ml, pH 8.0)[2]	0	no autotomy
ASW + pronase (10 mg/ml, pH 7.2)[2]	0	no autotomy

[1] Control for presence of APF activity following incubation period.
[2] Control for effect of proteolytic enzyme alone.

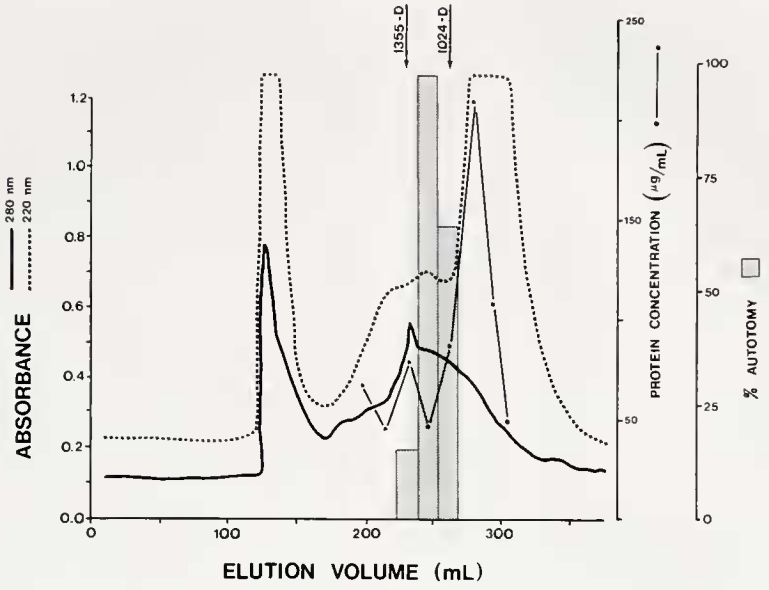

Figure 3. Elution pattern of crude autotomy-promoting factor on Sephadex G-25. Sample (100 ml) obtained from 1–2 sea stars was concentrated by freeze-drying and loaded in 3 ml on a Sephadex G-25 (fine grade) column (2.6 cm × 51 cm) equilibrated in 0.05 *M* NaCl at 4°C. Elution was carried out with the same solvent at a flow rate of 15 ml/h and 5 ml fractions collected. Fractions were pooled into 15 ml volumes and the protein content in each estimated by the method of Lowry. Each set of pooled fractions was then freeze-dried, redissolved in 3 ml of ASW and bioassayed (3 sea stars each). The column was calibrated with Blue Dextran 2000, vitamin B_{12} (MW = 1355), and angiotensin II (MW = 1046).

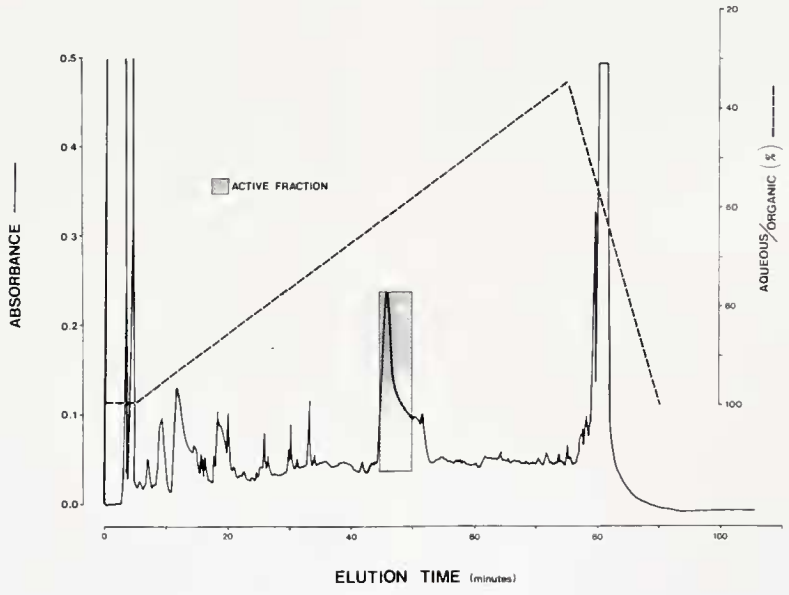

Figure 4. Typical reversed-phase HPLC elution pattern of Sephadex G-25 purified autotomy-promoting factor. An active fraction (15 ml) (elution volume 240–255 ml) from a column of Sephadex G-25 was freeze-dried, redissolved in water (1 ml) and loaded on a reversed-phase Supelcosil LC-18-DB column (250 mm × 4.6 mm, C_{18}, 5 μm particles) equilibrated in 0.1% trifluoracetic acid (aqueous solvent). After a 5-min run at 100% aqueous solvent, absorbed molecules were eluted at room temperature using a linear gradient of 100% to 35% aqueous solvent in 70 min at a flow rate of 1 ml/min. The organic solvent consisted of 0.1% trifluoracetic acid in 70% acetonitrile. Absorbance was monitored at 254 nm. Fractions (4 ml) were collected, vacuum centrifuged to dryness, redissolved in ASW (1 ml), and bioassayed (3 sea stars each). A major peak with autotomy-promoting factor activity (boxed and shaded area) was present. Mean time to initial arm autotomy was 2.5 min (S.E.M. = 0.5 min).

plex behavioral response that, after a few minutes, results in multiple arm autotomy, a generalized softening of the body wall, and morbidity. The pronounced response suggests that the amount of APF injected was an overdose.

APF from *P. helianthoides* is a polar, heat-labile substance. Fractionation by reversed-phase HPLC of Sephadex G-25 purified APF yielded a single peak of activity, indicating only a single component has APF activity. Gel permeation chromatography suggests that APF has a molecular weight of roughly 1200 Daltons. APF is ammonium sulphate precipitable, and its activity in crude extracts is reduced or destroyed by several proteolytic enzymes. It would thus appear to be a peptide or have a peptide component.

Little is known about other asteroid APFs. Chaet (1962) reported that the autotomy-promoting "toxin" from *Asterias forbesi* was a heat stable, dialyzable molecule that seemed to be derived from the parietal peritoneum or cells closely associated with it. *P. helianthoides* APF is derived from the body wall which, in our preparations, included the parietal peritoneum.

Fluids from scalded *Asterias vulgaris, A. forbesi, Solaster endeca* and *Crossaster papposus* are effective in promoting arm autotomy in both *A. vulgaris* and *A. forbesi* (Mladenov, in prep.). This suggests that APF-like molecules are present in many, if not all, sea stars and that they may have similar structures. However, unlike the *A. forbesi* "toxin," the activity of *P. helianthoides* APF is abolished after heating, indicating the probability of structural differences in the APF molecules of these particular species.

The evisceration factor from the holothurian, *Sclerodactyla briareus,* may be quite unlike *P. helianthoides* APF. Smith and Greenberg (1973) suggested that it is a very small, stable, molecule with none of the characteristics of a protein. The sources of this factor are likely to be peritoneal cells, cells within haemal vessels, or nerves associated with peritoneal or haemal tissues (Smith and Greenberg, 1973; Byrne, 1986). Chaet (1962) found that the *A. forbesi* toxin had no autotomy-promoting effect when injected into *Sclerodactyla briareus.*

The arms of *Asterias vulgaris* and *A. forbesi* are always autotomized at either side of the fifth pair of ambulacral ossicles (King, 1898; Anderson, 1956). Autotomy results from the sudden release of longitudinal muscles and connective tissues linking these ossicles, and by the softening and subsequent tearing of the tissues of the aboral body wall in the plane of breakage (Anderson, 1956). Arm autotomy in *P. helianthoides* is also facilitated by loss of tensile strength of the predominately collagenous aboral body wall at the arm base and of the collagenous structures linking the ambulacral ossicles (Wilkie, Emson, and Mladenov, in prep.). Autotomy in sea stars thus depends on the rapid loss of tensile strength of collagenous structures in the autotomy plane. Asteroid APF's must thus promote or regulate this process. The generalized softening of the body wall of *P. helianthoides* following APF injection is further evidence of this effect.

It is possible that stimulation of a sea star results in release of APF from cells in the body wall directly into the connective tissue matrix, thereby causing the softening response. Alternatively, APF may be released from the body wall into the coelom where it is transported to effector tissues. The presence of APF in the fluids of autotomized *P. helianthoides* is an indication that some APF is secreted into the coelom. Also, injection of APF into the coelom results routinely in autotomy. However, time to autotomy is longer following intracoelomic injection of APF (5.1 min, S.E.M. = 0.5 min, n = 10; this study) compared to time to autotomy following stimulation such as clamping the arm (0.4 min, S.E.M. = 0.1 min, n = 9; Mladenov, pers. obs.), which suggests that coelomic transport of APF may be incidental or secondary to direct release into the appropriate tissues.

Motokawa (1981, 1982a, b) isolated two heat-stable, low molecular weight factors from holothuroid coelomic fluid. One is methanol soluble and stiffens the connective tissue of the body wall; the other is methanol insoluble and softens the body wall. These interesting "softening" and "stiffening" coelomic factors have not been characterized, and their relationship to autotomy- and evisceration-promoting factors is unclear.

Many physiological lines of evidence implicate the nervous system in the control of the mechanical properties of echinoderm connective tissues (summarized in Motokawa, 1984, 1988; Wilkie, 1984, 1988). Morphological evidence of nervous control consists of the presence of granule-containing, neurosecretory-like cell processes in all mutable echinoderm connective tissues (Motokawa, 1984; Wilkie, 1984). Autotomy-promoting factors and coelomic "softening" and "stiffening" factors may thus prove to be neurochemicals. The physicochemical basis of variable tensility is unknown but may involve (1) changes in the charge distribution and conformation of the matrix macromolecules, (2) regulation of the extent of Ca^{++}-dependent electrostatic cross-bridging between anionic sites on matrix macromolecules, or (3) the formation of covalent cross-links between collagen fibrils and the matrix by means of an unknown Ca^{++}-dependent enzyme system (Motokawa, 1984, 1988; Wilkie, 1984, 1988; Diab and Gilly, 1984; Shadwick and Pollock, 1988). The complete characterization of APF may prove to be an important first step in resolving the physicochemical mechanism of variable tensility of echinoderm connective tissues.

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